AFRL-AFOSR-VA-TR-2020-0137

Regulatory Mechanisms of Radioresistance under Differential Levels of Ionizing Irradiation.

Lydia Contreras
UNIVERSITY OF TEXAS AT AUSTIN

08/12/2020
Final Report

Air Force Research Laboratory
AF Office Of Scientific Research (AFOSR)/ RTB2
Arlington, Virginia 22203
Air Force Materiel Command

DISTRIBUTION A: Distribution approved for public release.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ORGANIZATION.

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
06-14-2016	Final report	March 14, 2016-July 14, 2019

4. TITLE AND SUBTITLE

Regulatory mechanisms of radioresistance under differential levels of ionizing radiation

5a. CONTRACT NUMBER

5b. GRANT NUMBER

Grant FA9550-16-1-0174

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Lydia Maria Contreras, PhD

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Texas Austin
Austin, Texas, USA

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

not for public release

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Unraveling mechanisms of cell survival under oxidative stress is important to protect human health and increase human performance in harsh environments. As an extremophile that can withstand unusually high levels of ionizing radiation, Deinococcus radiodurans hides a wealth of strategies to manage oxidative stresses that are not fully understood. The overarching goal of this project was to characterize the function and biological importance of our newly discovered RNA regulators, in the context of ionizing radiation- and chemically-induced oxidative stress. We proposed to profile some of the most active candidates to investigate the new hypotheses that: (1) different mechanisms of oxidative stress management are triggered by different levels of radiation, and (2) that previously uncovered regulatory elements (i.e. 5'UTR) remain largely functional during oxidative stress to control transporters that are critical in initially boosting antioxidants while decreasing cellular toxins. The proposed work builds upon our recent discovery of 54 novel potential 5'UTR riboswitches that differentially regulate gene expression under relatively high (~15 kGy) and low (1 kGy) ionizing radiation and are largely found upstream of cellular transport genes.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(Include area code)*

INSTRUCTIONS FOR COMPLETING SF 298

1. REPORT DATE. Full publication date, including day, month, if available. Must cite at least the year and be Year 2000 compliant, e.g. 30-06-1998; xx-06-1998; xx-xx-1998.

2. REPORT TYPE. State the type of report, such as final, technical, interim, memorandum, master's thesis, progress, quarterly, research, special, group study, etc.

3. DATES COVERED. Indicate the time during which the work was performed and the report was written, e.g., Jun 1997 - Jun 1998; 1-10 Jun 1996; May - Nov 1998; Nov 1998.

4. TITLE. Enter title and subtitle with volume number and part number, if applicable. On classified documents, enter the title classification in parentheses.

5a. CONTRACT NUMBER. Enter all contract numbers as they appear in the report, e.g. F33615-86-C-5169.

5b. GRANT NUMBER. Enter all grant numbers as they appear in the report, e.g. AFOSR-82-1234.

5c. PROGRAM ELEMENT NUMBER. Enter all program element numbers as they appear in the report, e.g. 61101A.

5d. PROJECT NUMBER. Enter all project numbers as they appear in the report, e.g. 1F665702D1257; ILIR.

5e. TASK NUMBER. Enter all task numbers as they appear in the report, e.g. 05; RF0330201; T4112.

5f. WORK UNIT NUMBER. Enter all work unit numbers as they appear in the report, e.g. 001; AFAPL30480105.

6. AUTHOR(S). Enter name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. The form of entry is the last name, first name, middle initial, and additional qualifiers separated by commas, e.g. Smith, Richard, J, Jr.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES). Self-explanatory.

8. PERFORMING ORGANIZATION REPORT NUMBER. Enter all unique alphanumeric report numbers assigned by the performing organization, e.g. BRL-1234; AFWL-TR-85-4017-Vol-21-PT-2.

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES). Enter the name and address of the organization(s) financially responsible for and monitoring the work.

10. SPONSOR/MONITOR'S ACRONYM(S). Enter, if available, e.g. BRL, ARDEC, NADC.

11. SPONSOR/MONITOR'S REPORT NUMBER(S). Enter report number as assigned by the sponsoring/ monitoring agency, if available, e.g. BRL-TR-829; -215.

12. DISTRIBUTION/AVAILABILITY STATEMENT. Use agency-mandated availability statements to indicate the public availability or distribution limitations of the report. If additional limitations/ restrictions or special markings are indicated, follow agency authorization procedures, e.g. RD/FRD, PROPIN, ITAR, etc. Include copyright information.

13. SUPPLEMENTARY NOTES. Enter information not included elsewhere such as: prepared in cooperation with; translation of; report supersedes; old edition number, etc.

14. ABSTRACT. A brief (approximately 200 words) factual summary of the most significant information.

15. SUBJECT TERMS. Key words or phrases identifying major concepts in the report.

16. SECURITY CLASSIFICATION. Enter security classification in accordance with security classification regulations, e.g. U, C, S, etc. If this form contains classified information, stamp classification level on the top and bottom of this page.

17. LIMITATION OF ABSTRACT. This block must be completed to assign a distribution limitation to the abstract. Enter UU (Unclassified Unlimited) or SAR (Same as Report). An entry in this block is necessary if the abstract is to be limited.

Grant/Award #: FA9550-16-1-0174

PI Name: Lydia M. Contreras

Organization/Institution: University of Texas-Austin

Project Title: Regulatory mechanisms of radioresistance under differential levels of ionizing radiation

Final Report- Year 3 (Fiscal Year 2019)

August 11, 2020

I. Project Abstract (Same as reported during the first two years of the report)

Unraveling mechanisms of cell survival under oxidative stress is important to protect human health and increase human performance in harsh environments. As an extremophile that can withstand unusually high levels of ionizing radiation, *Deinococcus radiodurans* hides a wealth of strategies to manage oxidative stresses that are not fully understood. A major gap is exclusion of gene expression regulation on all current radioresistance models; as a result, a "one fits all" activation mechanism is assumed for all oxidative stress recovery pathways, despite levels of exposures.

The **overarching goal** of this project was to characterize the function and biological importance of our newly discovered RNA regulators, in the context of ionizing radiation- and chemically-induced oxidative stress. We proposed to profile some of the most active candidates to investigate the ***new hypotheses*** that: (1) different mechanisms of oxidative stress management are triggered by different levels of radiation, and (2) that previously uncovered regulatory elements (i.e. 5'UTR) remain largely functional during oxidative stress to control transporters that are critical in initially boosting antioxidants while decreasing cellular toxins. The proposed work builds upon our recent discovery of 54 novel potential 5'UTR riboswitches that differentially regulate gene expression under relatively high (~15 kGy) and low (1 kGy) ionizing radiation and are largely found upstream of cellular transport genes.

Specifically, we proposed: **(i)** characterization of the activation of our 5'UTRs under different levels of radiation- and chemically-induced oxidative stresses, **(ii)** characterization of the biological relevance of the most active 5'UTR regulators to the radioresistance phenotype, and **(iii)** screening of specific metabolites of relevance to oxidative stress management through their interactions with functionally significant 5'UTR regulators.

We ultimate expect this work to contribute to harvesting *natural* RNA sensors that inspire new scaffolds for the detection of compounds of relevance to the Air Force Intelligence Surveillance and Reconnaissance efforts.

II. Major Goals of the entire project (Years 1-3)

The immediate goal of this research was to characterize native regulatory parts that are differentially activated within *D. radiodurans* radioresistance. While our initial work (funded by an AFOSR Young Investigator Award) focused on large discovery experiments, this three-year project the work focused on detailed mechanistic characterization of the most biologically relevant regulatory RNAs that we have uncovered. Our ultimate goal was to advance mechanistic knowledge of pathways that are regulated under various levels (and types) of oxidative stresses. Our focus on ionizing radiation has been inspired by the challenge of finding biological systems that can survive these types of environmental conditions and that can therefore serve to understand effective pathways used in fighting general cellular oxidative stresses.

Understanding of regulatory strategies that benefit oxidative stress management will contribute new potential therapeutic measurements to protect human health and increase human performance in harsh environments. Furthermore, knowledge of the regulatory parts and regulated pathways (in a way that depends on radiation levels) will be applicable to the general efforts of addressing current limitations in engineering robust, controllable and diverse synthetic platforms (e.g. sensors) that respond to physical stimuli.

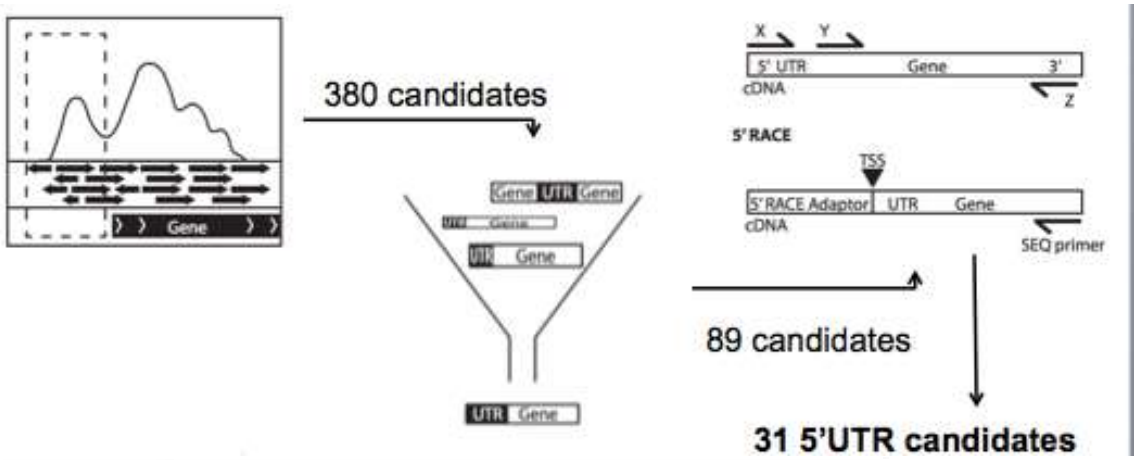
A total of three aims were proposed in this work and they were completed to the following extend:
Aim 1 (proposed for year 1): Characterization of the activation of the identified 5'UTRs under different radiation- and chemically induce oxidative stresses. **100% was completed**

Aim 2 (Proposed for Year 2): Characterization of the biological relevance of the most active 5'UTR regulators to the radioresistance phenotype. **100% of was completed**

Aim 3 (Proposed for Year 3): In vivo functional mechanisms that underlie radiation responsive RNA candidates of interest. We completed ~75% of this goal; little progress was made in terms of the ligand screening for the riboswitches given that the mechanisms that underlie function of our most interesting candidates did not seem to depend only on ligand binding but also appeared to depend on the presence of cellular co-factors). These mechanisms were not easily captured in vitro.

III. Most significant Accomplishments during the four year duration of the project (Note: an option year was awarded for this project*). The following major results and outcomes have resulted from this project.

1. *Reanalysis of all the original 54 candidates identified for improved filtering of our initial target list.* We revisited our data for more strenuous analysis of all the information we had collected on 54 regulatory candidates to further narrow down the list for follow-up experiments. Specifically, we re-analyzed all of our transcriptome data sets and our 5'UTR candidates to apply more thresholds. In this new analysis, (a) we remove any ribosomal proteins and tRNA synthatases, (b) we take into account transcript counts (remove candidates with less than twice the average read per genome nucleotide (3.25), and (c) we favor metabolic enzymes or those previously described in proteomics literature as being unregulated after IR. We obtain 31 candidates listed below (which were used for the screens and studies originally proposed).



DR_#	Gene Name
2221	tellurium resistance protein TerD
640	Methionine adenosyltransferase
2208	lactylglutathione lyase
2630	thyA thymidylate synthase
378	TetR family transcriptional regulator
1617	xanthine phosphoribosyltransferase
129	dnaK
349	ATP-dependent protease LA
1247	sucC succinyl-CoA synthase subunit beta
561	maltose ABC transporter periplasmic maltose-binding protein
2361	acyl-CoA dehydrogenase
302	glucosamine-fructose-6-phosphate aminotransferase/glmS switch
1778	3-isopropylmalate dehydratase large subunit
2438	endonuclease III
1771	uvrA
694	Hypothetical protein
1913	DNA gyrase
2569	Hypothetical protein
1356	Transporter cluster
139	GTP-binding protein HflX
950	NADH Dehydrogenase
1768	Hypothetical protein
971	Electron transfer flavoprotein subunit beta
1279	Mn superoxide dismutase
2588	Iron ABC transporter periplasmic substrate-binding protein
1573	pyrG CTP synthase
899	Ribonuclease H
153	riboflavin-specific deaminase/FMN switch
1817	phospho-2-dehydro-3-deoxyheptonate aldolase
309	Elongation factor Tu
1987	Hypothetical protein

2. We Identified 5'UTR candidates that activated gene expression in response to various levels of ionizing radiation levels; these can serve as regulatory "parts" in synthetic circuits. We identified the following 5'UTR candidates that affect gene expression post exposure to the **lower ranges tested ~1kGy**: **(1)** DNA gyraseA (DR1913) and (2) ATP protease (DR349). We also identified

two other ones that affect gene expression post exposure **to more acute higher radiation levels tested (10kGy). (2)** Mn superoxide dismutase (DR1279), **(3)** DNA gyraseA (DR1913). Importantly, these candidates did not respond to any H_2O_2. stress tested. Instead, we found that a different unique UTR (DR1857, OHRP) uniquely responded to 25mM H_2O_2.

These experiments were done by exposing *D. radiodurans* harboring each individual candidate from the library of all 54 newly discovered 5'UTRs* to varying levels of ionizing radiation, hydrogen peroxide and Paraquat. We confirmed irradiation consistency through survival curves, quantification of double strand DNA breaks and overexpression of the RecA repair enzyme. Evaluation of the activation potential of the 5'UTR library, as measured by changes in GFP levels. We irradiated cells transformed with the 5'UTR-GFP plasmids (all the candidates included in the table above). Specifically, conducted radiations under 0, 1, 3, 5, 7, 9, and 12 kGy with a 10 MeV, 18 kW LINAC (linear accelerator) gamma-ray source at the National Center for Electron Beam Research, a public facility at Texas A&M University, as we have previously done using doses of 250Gy/s. All exposures were confirmed by running western blotting analysis to evaluate changing levels of RecA on our samples to check expected levels of oxidative stresses that we have characterized in the past.

Some of these results were also published as part of a review of bacterial small RNAs and riboswitches **. Moreover, during a few technical meetings, we also interacted with Dr. Howard Salis and Dr. Warren Powell about building mathematical models for our regulators (after learning what each of these two investigators worked on); two manuscripts resulted from these interactions (***, ****).

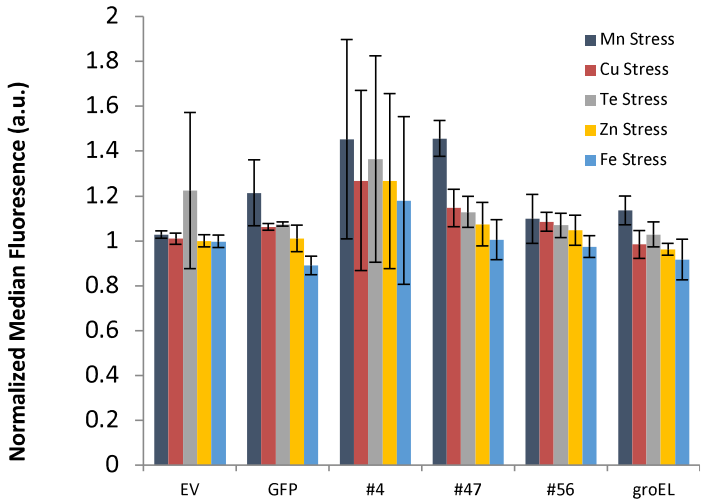
*Genome-wide search for ionizing radiation responsive 5'UTRs in Deinococcus radiodurans reveals post-transcriptional regulation in the Radiation and Desiccation Response (RDR) for DNA gyrase subunit A," *Applied and Environmental Microbiology*, 83(12): e00039-17 (2017). Available from: doi:10.1128/AEM.00039-17.

** J. K. Villa, Y. Su, **L.M. Contreras** and M. Hammond. "Synthetic Biology of Small RNAs and Riboswitches", *Book chapter*, in Papenfort, K, Storz, G (eds.), Regulating with RNA in Bacteria and Archaea. ASM), 6(3) (2018). ASM. Available from: doi.org/10.1128/microbiolspec.RWR-0007-2017.

*** A. N. Leistra, G. Gelderman, S. W. Sowa, A. Moon-Walker, H. M. Salis, and **L.M. Contreras**. "A Canonical Biophysical Model of the CsrA Global Regulator Suggests Flexible Regulator-Target Interactions" *Scientific Reports*, 8:9892 (2018). Available from: doi.org/10.1038/s41598-018-27474-2.

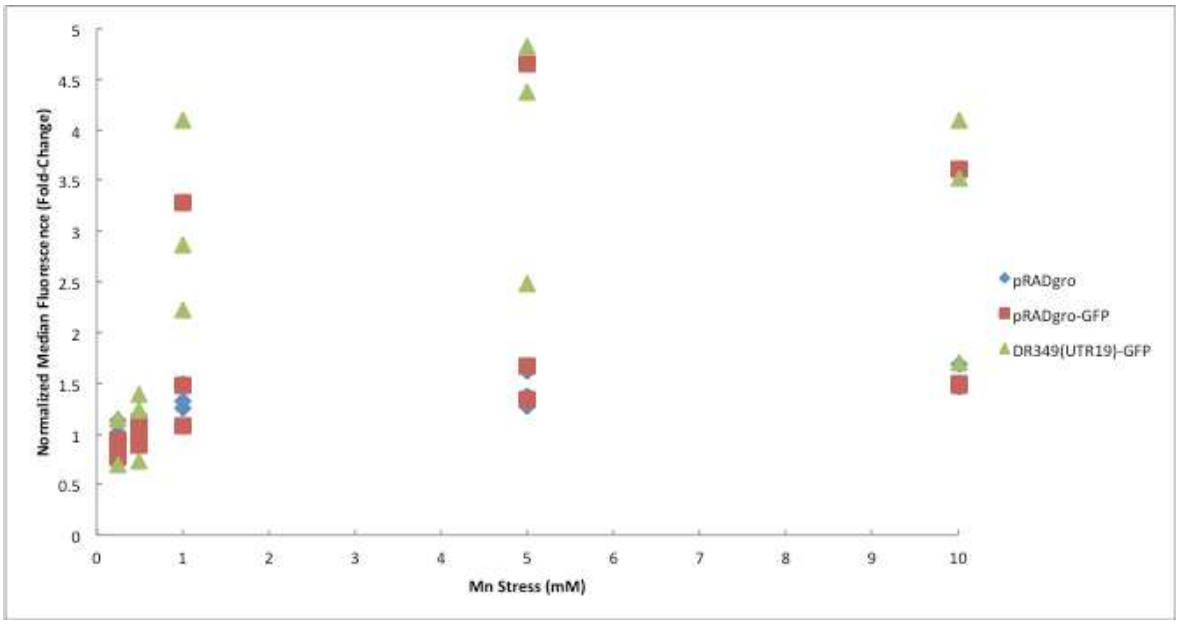
**** M. K. Mihailovic, J. Vazquez-Anderson, Y. Li, P. Vimalathas, V. Fry, R.A. Lease, W. Powell, and **L.M. Contreras**. "High-throughput in vivo mapping of RNA accessible interfaces to identify functional sRNA binding sites." *Nature Communications*, 9:4084 (2018). Available from: doi.org/10.1038/s41467-018-06207-z.

3. Identified 5'UTR candidates that differentially activated gene expression based on different levels of intracellular metal concentrations. We tested all 41 candidates under 500µM $MnCl_2$, 20 mM $MnCl_2$, 500µM $CuCl_2$, and 40µM K_2TeO_3 As shown in the case of the ATP protease 5'UTR (candidate #47, shown below under various levels of MnCl2), levels of gene regulation showed a dependence on intracellular concentrations of metals.

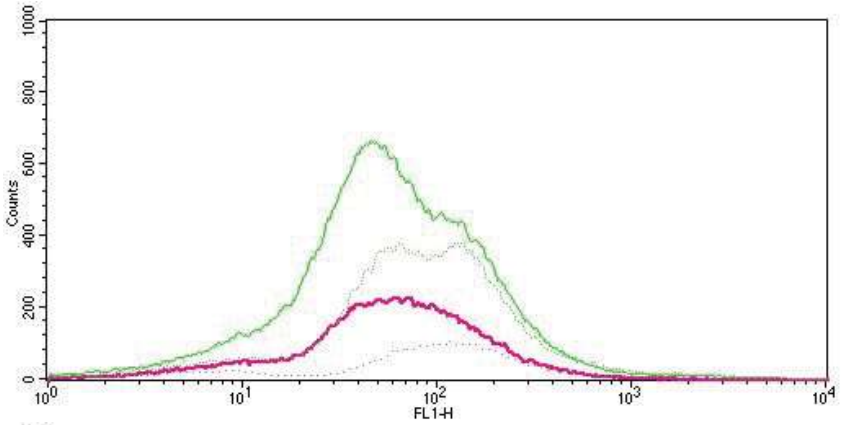


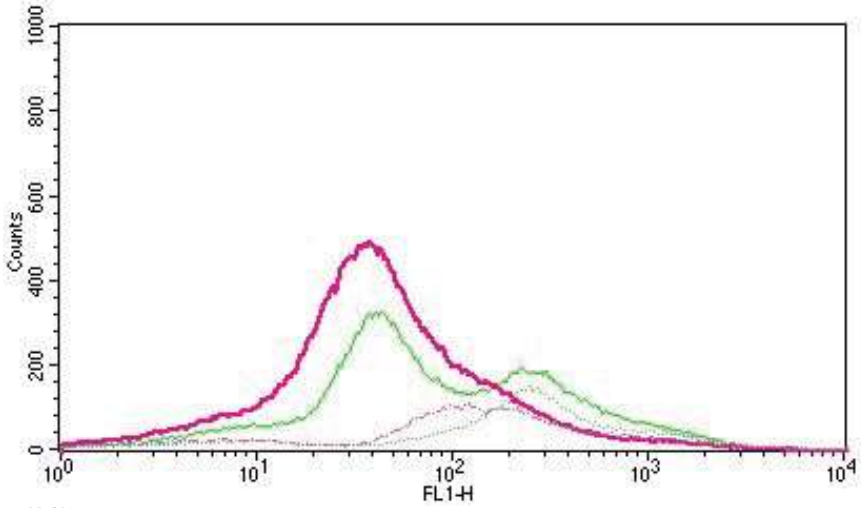
2hr time point with 500µM Mn, Cu, Zn, Fe and 40µM Te

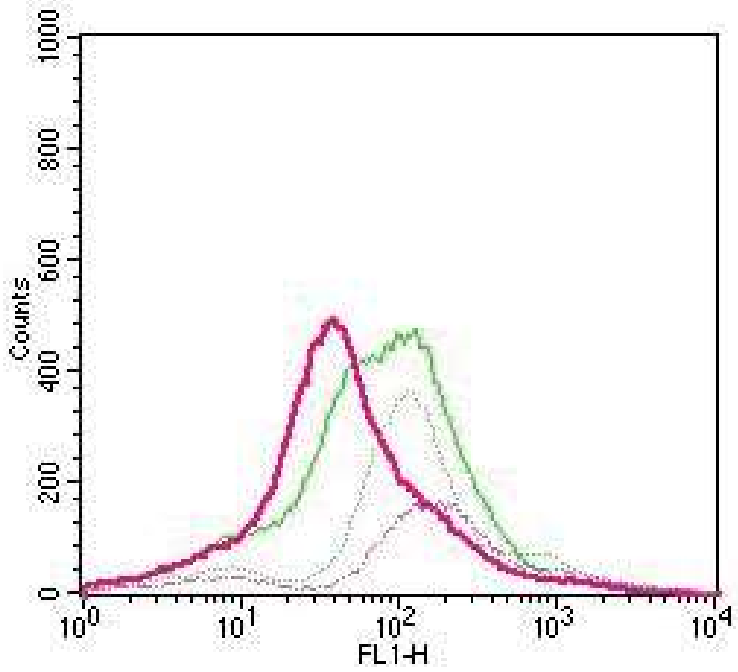
Basal Fluorescence Levels of promoters (GFP -pRADgro) and groEL are theoretically the same promoter) and the normalized fold-change (value 1=no change in median fluorescence after metal addition). These are samples for multiple 5'UTRs where the background levels of pRAD-GRO GFP fluorescence prevent acquisition of highly significant changes in fluorescence as a result of the 5'UTR under metal stress. EV is a control "Empty Vector" (showing background levels of fluorescence), and GFP is a positive control, where GFP is in the vector, in the absence of any 5'regulatory UTR element.



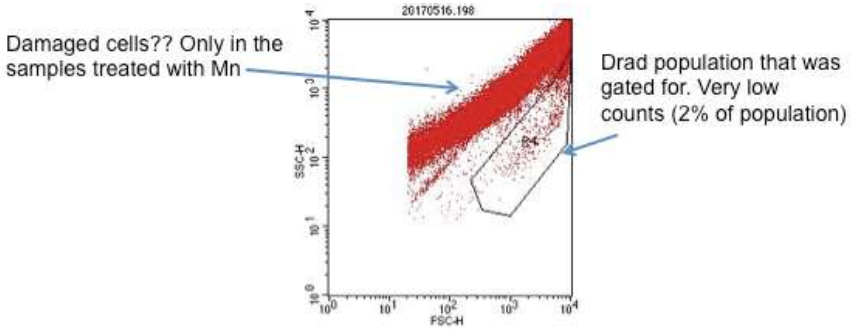
Histograms from 10mM data set (each graph is a different biological replicate set).
Green= pRADgro-GFP
Pink=UTR19(ATPprotease UTR)
Solid=Untreated
Dashed=10mM treated







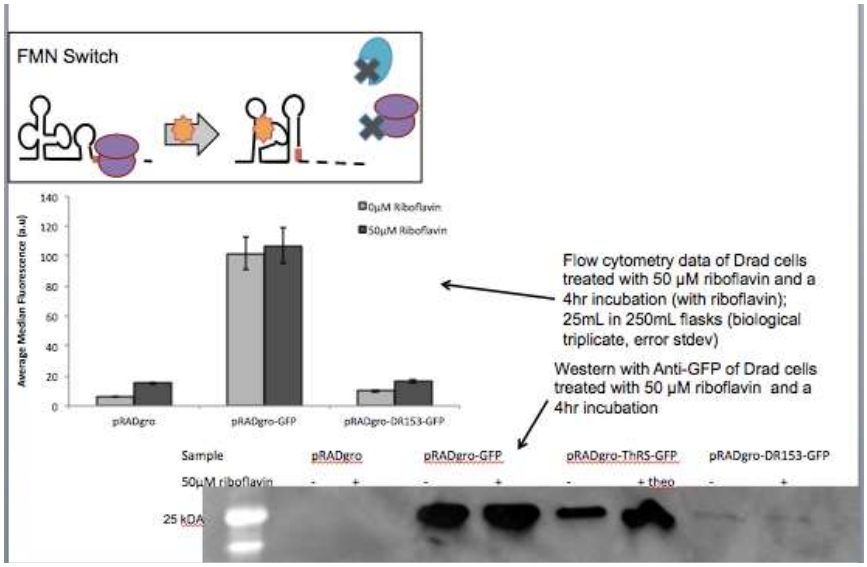
It is worth noting that significant time and effort was spent working around issues of cell toxicity (shown below) and metal ppt as we optimized metal concentrations to use for our screens. Because the living population of Drad is so low, there was a lot of initial error in the measurements we took, delaying our progress significantly. We also had the realization that the background levels of pRadGro were not as robust to evaluate activity of all our candidates. This prompted the design and validation of a new reverse reporter, described below (which was not initially planned in the proposed experiments). The construction of a new reverse reporter aimed to report on UTR gene repression, independently from the noise of pRADGRO plasmid as we discovered that the GRO promoter can be sensitive to metal stress.



4. <u>First attempt failed to establish novel in vivo assay to measure 5'UTR-mediated downregulation of gene expression in *D. radiodurans: inability to validate it and challenges of the pGRO promoter responding to the stresses (independently of the 5'UTR activity being tested)*</u>

   A challenge in the assay used for the above experiments was that the pGRO promoter is (on its own) also responsive to some of these metal stresses); so we had to tightly control for background effects. As a result of this we redesigned and implemented a more robust system (see below) that is also capable of measuring gene expression repression. The system that we used earlier was much more efficient at measuring gene activation but could not capture well gene repression effects.

   The new system developed as part of this work sought to evaluate down/up regulatory effect of the identified 5'UTRs on gene expression; specifically, it was designed for detection of lower activity (i.e. down regulation). As a proof of principle, we attempted optimizing down regulation with the FMN switch (DR153), which we had experimentally confirmed in *D. radiodurans.* The 5'UTR DR153 is a riboswitch that upon binding flavin mononucelotide results in the repression of transcription and translation of the downstream coding sequence. Previous studies in *E.coli* had seen in vivo repression of luciferase by FMN switch with addition of riboflavin. This experiment was attempted with *D. radiodurans* with the FMN 5'UTR-GFP construct. Unfortunately, we discovered that Drad does not naturally contain a flavin importer***. Therefore as shown below this experiment was unsuccessful. We therefore were never able to identify a clear proof of concept to evaluate the dynamic range of our fluorescence reporter for down regulation. The data included below summarizes these results.



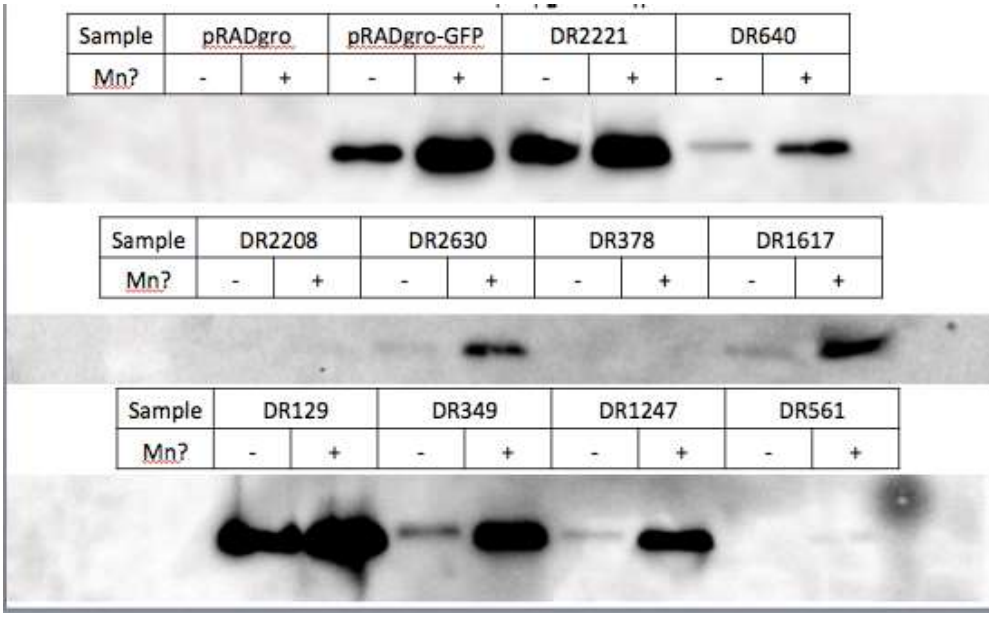
***Refs:

(1) *Yang P, Chen Z, Shan Z, Ding X, Liu L, Guo J. 2014. Effects of FMN riboswitch on antioxidant activity in Deinococcus radiodurans under H2O2 stress. Microbiol Res 169:411–416;*

(2) Pedrolli D, Langer S, Hobl B, Schwarz J, Hashimoto M, Mack M. 2015. The ribB FMN riboswitch from Escherichia coli operates at the transcriptional and translational level and regulates riboflavin biosynthesis. FEBS J 282:3230–3242;

(3) Gutiérrez-Preciado A, Torres AG, Merino E, Bonomi HR, Goldbaum FA, García-Angulo VA (2015) Extensive Identification of Bacterial Riboflavin Transporters and Their Distribution across Bacterial Species. PLoS ONE 10(5): e0126124. doi: 10.1371/journal.pone.0126124

As shown below, we tested some candidates via Western bloting to see possible down regulation (might permit detection of GFP levels too close to the pRADgro fluorescence). All pRADgro-DR#-GFP candidates blotted with anti-GFP; samples treated with 500μM $MnCl_2$ for 6hrs; protein lysed using sonication (10W 1minx3 on ice) and total protein measured with Bradford, 30μg loaded)). This data could not be interpreted well as we ran into the issue that the pRADgro promoter itself is sensitive to these conditions.



.

5.Established novel in vivo assay, independent of the pRAD-GRO promoter typically used in *D. radiodurans* to detect in vivo 5'UTR downregulation using the GFO promoter: a reverse reporter

We developed a new assay (based on a previously published reporter in Bacillus) to better determine and characterize UTRs that regulate via down regulation mechanisms (the more common mechanism that is difficult to capture in our current system). This reverse reporter uses the changes in expression of the repressor protein to measure UTR-based regulation via a reporter gene under control of the repressor protein regulated promoter. In this way, any regulation of the UTR is observed via an increase in fluorescence. Our expectation was that this assay better enable detection of loss-of-fluorescence approaches in Drad.

To utilize this new system in Drad, several parts to this system were optimized. These parts include:

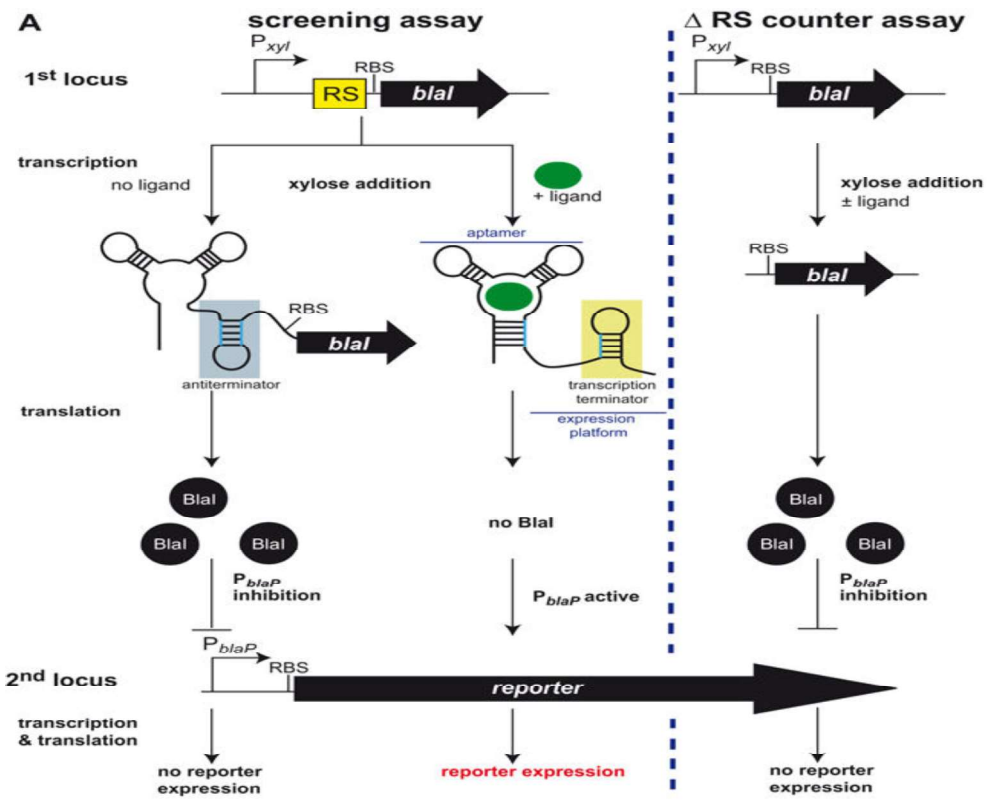
-Inducible promoter: (pSpac promoter, lac repressor based promoter (IPTG inducible); this has been utilized in Drad before (p11530 plasmid) but not in this reporter context

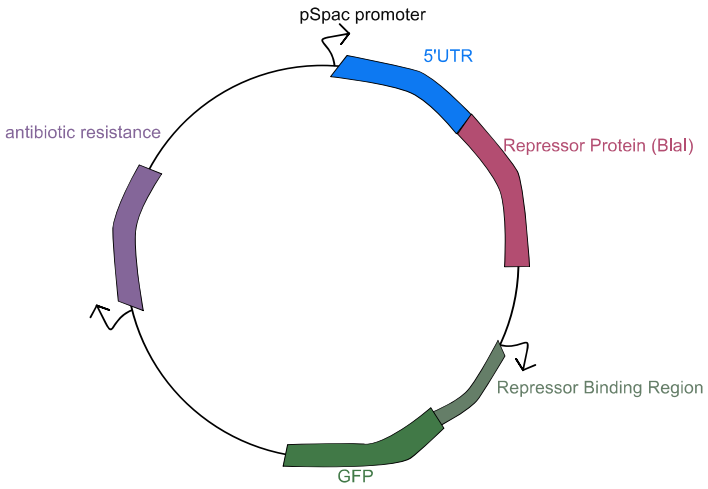
-Repressor protein BlaI: this is a repressor protein from Bacillus (which genes/proteins from Bacillus have been used in Drad before including the pSpac promoter) Repressor-binding promoter BlaP: BlaI binds this and represses transcription

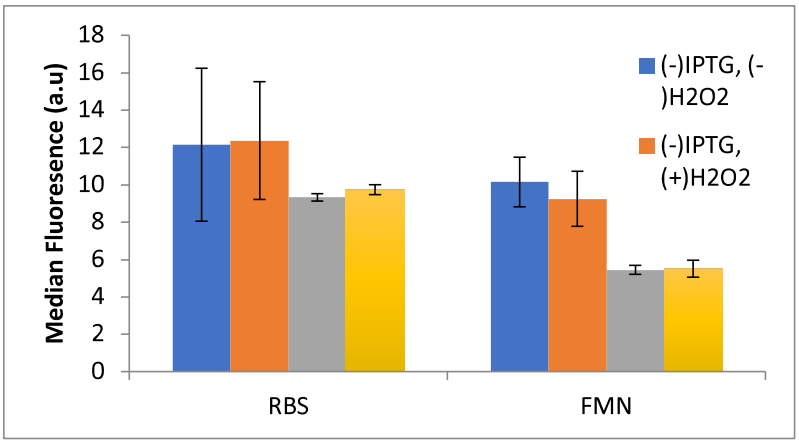
-GFP: this has already been optimized for Drad.

To optimize the above parts we designed the following constructs for use in Drad:
BlaP-GFP: to test the strength of the promoter in Drad to determine max signal
pSpac-GFP: to test induction times and concentrations for max signal
BlaI-His: to run western blot to confirm BlaI expression in Drad.

Two reverse reporters were made using the RBS only (no UTR control) and the FMN riboswitch (as a possible regulatory UTR). As shown below, we were able to differentiate the signal between non-UTR containing plasmids and UTR containing plasmids (with the FMN control) under stress.







Reverse reporter was used to rescreen all 5'UTR candidates but no 5'UTR that contributed to gene downregulation was identified. It is worth noting that we spent a major amount of time working around issues of plasmid optimization as many of the new plasmids that we have to use in *D. radiodurans* (to achieve an inducible system required major optimization). **A major accomplishment of this work was the establishment of all the assays and genetic tools that we need to complete characterization of some of our most interesting regulatory RNAs that are important to radiation survival**.

6. We optimized gene deletion protocols in *D. radiodurans*. We transition initial protocols from our work with Dr. Roland Saldahna (at the AFOSR labs) into our own laboratory and optimized these protocols for gene deletion of small RNAs and short regulatory RNAs. Briefly, to delete 5'UTR regions from the genomes of *D. radiodurans*, we used a suicide plasmid to introduce the desired interruption by homologous recombination. The upstream and downstream homologous regions were cloned into pUC19mPheS plasmid alone with kanamycin resistance sequence and lox66 and lox71 sequences. Then the plasmid was transformed into *D. radiodurans* R1 for double crossover homologous recombination using a previously reported protocol. *D. radiodurans* R1 were grown to late log phase (optical density at 600 nm [OD600] = 1) and mixed with 30 mM $CaCl_2$ and 10% glycerol to gain competence. Cells were then incubated on ice with plasmid DNA for 30 mins and then at 30°C for an hour. Fresh TGY medium was added to transformed cells for overnight incubation and the cells were plated on kanamycin plates (16 µg/ml) for selection. Plasmid pDeinoCre, which can express the Cre recombinase, was transformed into the same cells to remove the kanamycin resistance region from the genome by Cre-Lox recombination. Since *D. radiodurans* has multiple copies of its genome, we have found it often necessary to restreak on the plate with kanamycin for several rounds bring to homozygosity. When it proved impossible to purify mutant from wild-type, we assumed that the 5'UTR is essential and the mutant can only be maintained if a wild-type copy is also present.

7. Discovery and characterization of 8 new promoters for pathway engineering and heterologous protein expression in *D. radiodurans*. In summary, only a few plasmids, promoters, etc. that are highly controllable (i.e. inducible) exist in Drad, limiting the genetic

manipulation of this non-model organism. In this work, we uncovered 8 novel native *D. radiodurans* promoters that result in different levels of expression and construct minimal versions of these promoters to facilitate their use in biotechnology applications within this host organism. A highlight of this work is that a few of these promoters show comparable or higher expression to pGRO_ES, the most well used promoter in *D. radiodurans* research. Our hope is that this work now facilitates the constructions of new protein systems and pathways in this organism. In addition to what was published, we attempted a few experiments (that were not successful) that included antisense knockdown of the essential gene recA to test the different silencing abilities of the various promoters in the context of a gene that is easily measurable by viability phenotypic assays. Unfortunately, these experiments were not successful as the cloning of the gene continuously failed. Three strategies were attempted: (1) a full gene antisense design, (2) a full antisense targeting upstream (175bp) to 90 bp into the gene, and a (3) antisense design targeting the regulatory region of the operon (500bp upstream of operon).

A second series of experiments that was also unsuccessful was the implementation of microscopy to investigate the heterogeneity of these promoters. Specifically, we attempted time-lapse microscopy with Dr. Vernita Gordon's lab to identify patterns potentially involved with heterogeneity. These experiments were attempted twice for 4 hrs but couldn't get the amount of signal/resolution we needed to track and analyze single cells. We determined that growth in the plate was a problem since D. *radiodurans* doesn't attach and stay in the same place.

This published work is:.
A. Chen, M.W. Sherman, and **L.M. Contreras**. "Discovery and Characterization of Native *Deinococcus radiodurans* Promoters for Tunable Gene Expression". *Applied and Environmental Microbiology, AEM.01356-19 (2019).* Available from: doi.org/10.1128/aem.01356-19 **Paper featured in issue's cover.**

9. Developed an In vivo sensing system (IRS3) optimization for riboswitches in *D. radiodurans* for summer visit to Dr. Benavides-Chavez's lab (Airforce lab). Our goal here was to develop intracellular structural probing tools to characterize functional states of evolved RNA sensors (i.e. riboswitches). We have developed a number of synthetic biology applications to understand function-structure relations of designed/engineered RNA sensors (these have been published in NAR and Nature Communications for the characterization of other types of RNA regulators in vivo). We have started working with Dr. Benavides-Chavez and his group to understand if we can adapt our structural-sensing tools (IRS3) and INTERFACE) to test 5'UTR binding to aptamer (characterize structural changes upon binding). This will be tested in *E. coli* first and optimized to cell-free second. This will allow us screening of the binding capabilities of our own 5'UTRs.

As outlined below, a question that we aimed to examine was: can we use IRS3 to determine riboswitch structural changes after binding ligand? We designed this system starting with the well-characterized ThRS attached to mStrawberry. Theoretically, this was to give both accessibility of the Theophylline riboswitch (ThRS) AND a report of Theophylline riboswitch activation. In the last couple of months, we have cloned the initial reporter plasmid with 7 probes designed across the sequence space of ThRS. Testing with ThRS will begin shortly in *E.coli* K12.

We designed and tested the system over the summer (My student Jo Villa was a summer fellow in his lab). Some iterations to the design were made, based on initial results obtained at the Airforce labs in the context of the ThRS riboswitch in *E.coli* and a model TNT riboswitch from the lab; **this project was deprioritized and severely affected by the COVID shutdown (we are currently still testing the new designs).**

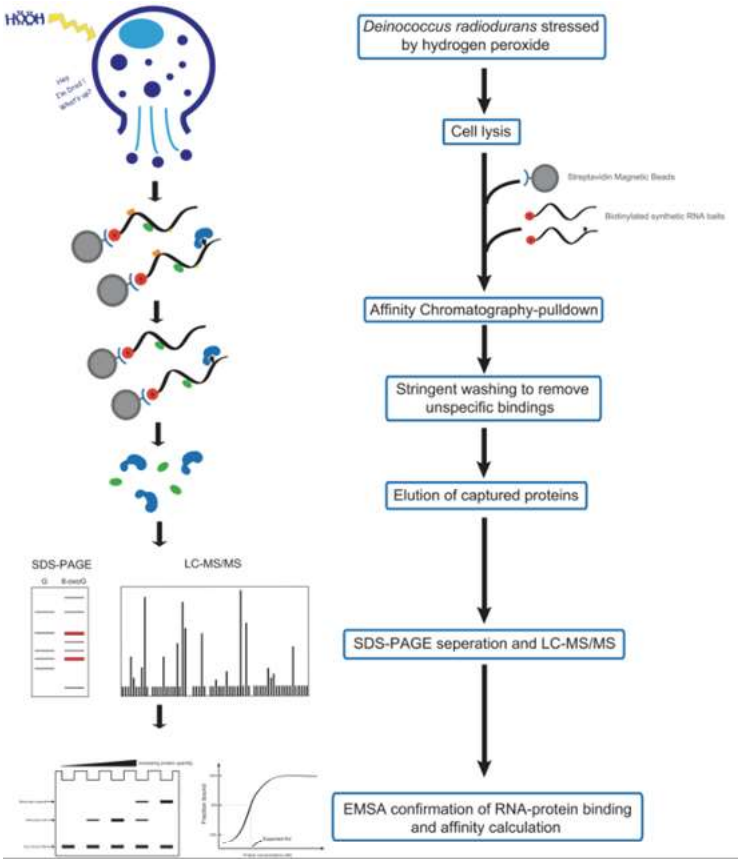
8. Identification and characterization of novel 8-oxoG RNA modification binding proteins in Deinococcus radiodurans.

A question that arose in our research, while characterizing novel regulatory RNAs is whether these RNAs use chemical modification (like 8OGs) as sensory marks that change their chemistries to better interact with other cellular factors that are also involved in oxidative stress responses. As part of investigating this potential new mechanistic chemical feature, we developed an assay to pull down *D. radiodurans* native proteins that interacted with RNAs that contained 8OG; 8OG is an oxidative chemical mark on the guanosine of a nucleic acid and it is the most well-known chemical mark of oxidative stress (at the molecular level).

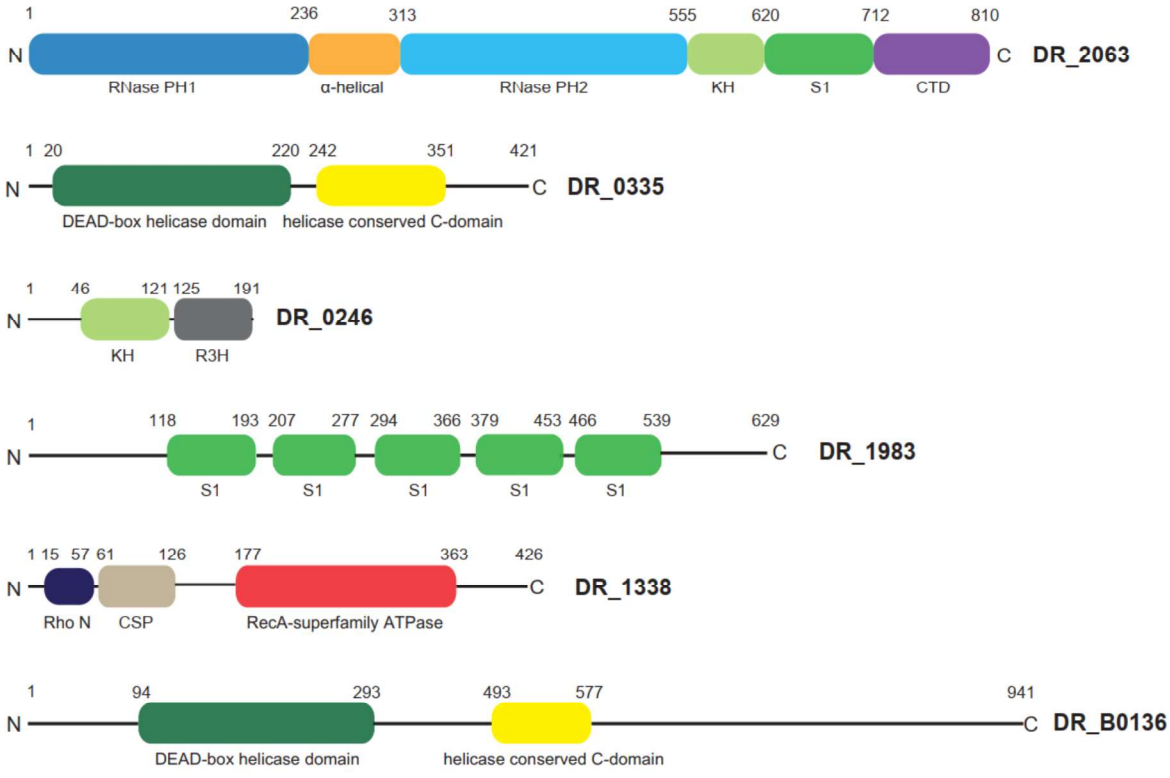
As illustrated below, for these experiments, we prepared lysates from *D. radiodurans* cells by sonication, centrifugation and concentrated via Amicon Ultra-2 Centrifugal Filter Unit. We pre-cleared the lysates with streptavidin-conjugated beads and yeast tRNA to reduce the nonspecific binding. Then, we incubated the biotinylated RNA baits with pre-cleared cell lysates. The mixture was then added to streptavidin-conjugated beads pre-blocked with BSA and tRNA. RNA-protein complexes were pulled-down and washed extensively. The eluted proteins were separated on SDS-PAGE gels and stained with SYBRO Ruby stain. The remaining elution was sent for LC-MS/MS analysis. Three independent biological replicates were performed. The following seven proteins were identified (shown in the table below). These seven proteins harboring RNA-binding domains were identified to be enriched in the pull-down elution with 8-oxoG modified RNA.

Lastly, we have overexpressed all these seven proteins in vectors, purified them and run in vitro binding assays (EMSAS) with chemically modified 24-mer 8-OG modified RNAs to validate that they indeed bind and recognize RNAs that have been chemically modified with this oxidative stress mark. The sequences used were: NNGNNNGNNNGNNNGNNNGNNNGN [(NNGN) X6] (unmodified) and NNoxoGNNNoxoGNNNoxoGNNNoxoGNNNoxoGNNNoxoGN [(NNoxoGN) X 6](modified). Specifically, for these experiments, we synthesized unmodified RNA and 8- vectors, purified them and run in vitro binding assays (EMSAS) with chemically modified 24-mer 8-OG modified RNAs to validate that they indeed bind and recognize RNAs that have been chemically modified with this oxidative stress mark. The sequences used were: NNGNNNGNNNGNNNGNNNGNNNGN [(NNGN) X6] (unmodified) and NNoxoGNNNoxoGNNNoxoGNNNoxoGNNNoxoGNNNoxoGN [(NNoxoGN) X 6](modified). Specifically, for these experiments, we synthesized unmodified RNA and 8- oxoG modified RNA were radiolabeled and purified. The labeled RNAs were incubated with different purified protein (His-labeled purified from BL21 E.coli) candidates. The RNA-protein mixtures were denatured for 5 min at 70 C, then incubated at 37C for 1.5h. Samples were run for 4 h at 25–35 mA in a 5% non-denaturing polyacrylamide gel with 0.5X TBE running buffer. The gel was dried at 80 C for

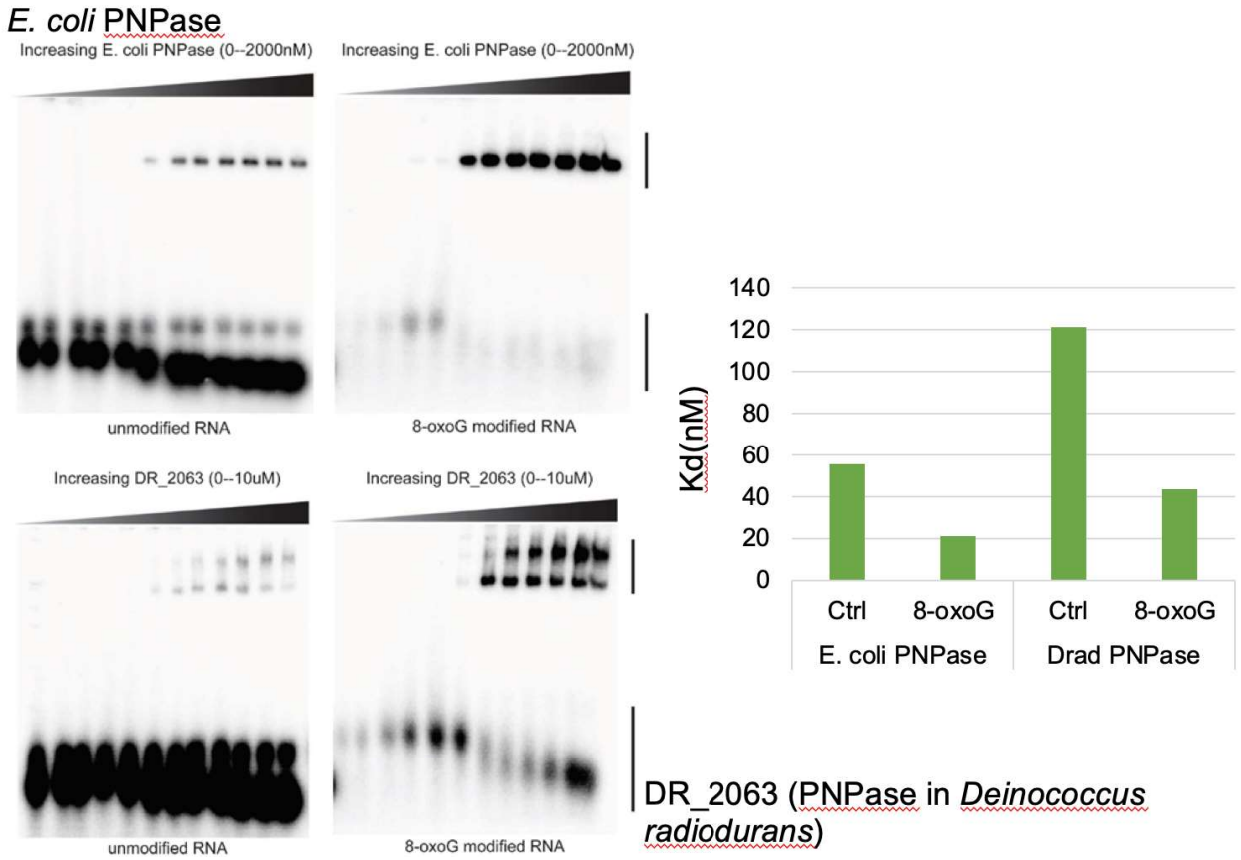
1.5 h and phospho-imaged using Typhoon FLA 700. (Note: DR_B0136 couldn't be expressed and purified efficiently).



Identified Proteins	Protein ID	Enrichment (8-oxoG counts/unmodified counts)					
		Sham			H2O2		
		Replicate A	Replicate B	Replicate C	Replicate A	Replicate B	Replicate C
Polyribonucleotide nucleotidyltransferase	DR_2063/Pnp	0.75	**2.57**	1.25	0.93	1.26	1.13
Transcription termination factor Rho	DR_1338/Rho	**2.94**	**3.96**	**2.01**	1.29	**3.61**	1.09
Nucleic acid-binding protein, putative, HRDC family	DR_2444	0	0.82	∞	0	0.40	**2.19**
ATP-dependent RNA helicase	DR_0335	1.17	0.95	1.14	**5.16**	1.45	1.21
Ribosomal protein S1	DR_1983	**5.51**	0.30	**2.17**	**3.78**	0.28	1.88
ATP-dependent helicase HepA	DR_B0136	0	**2.26**	0.76	∞	**4.82**	0.21



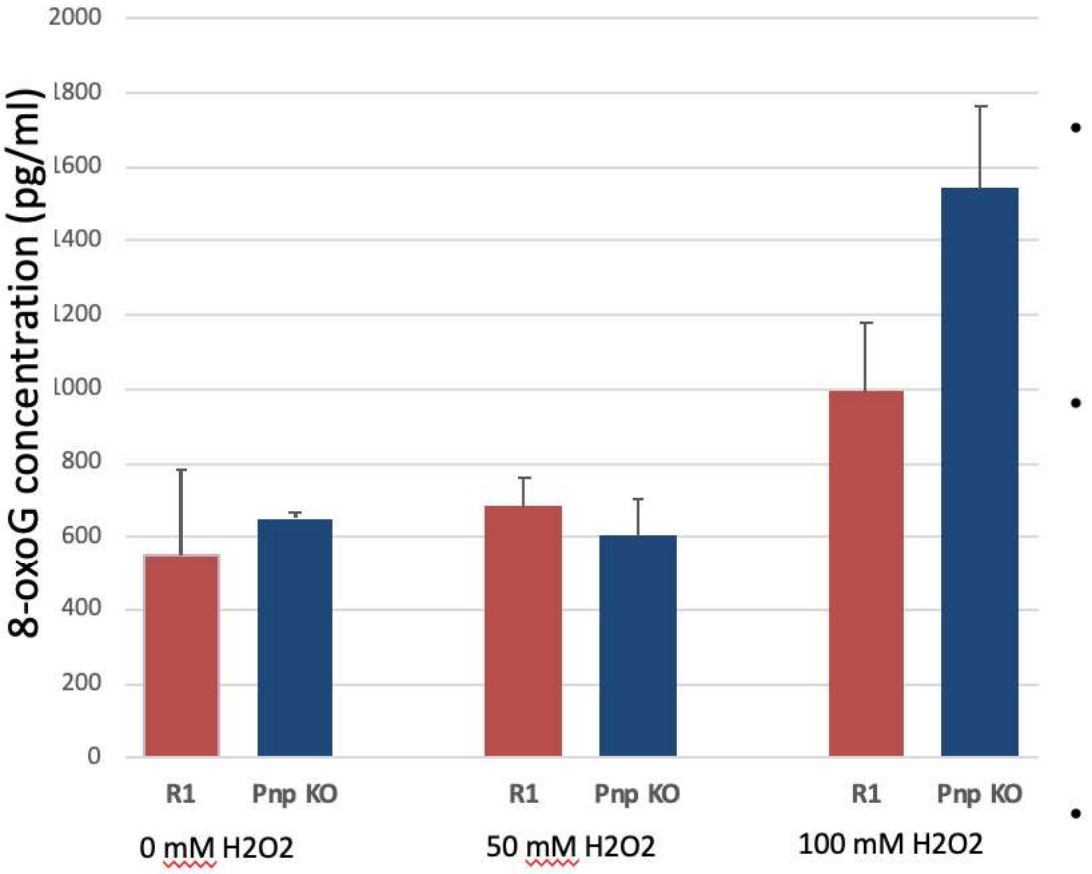
One of the discoveries of this work is that a well conserved protein in bacteria (PNP, DR_2063) has a similar preference to 8-oxoG modified RNA as E. coli PNPase but has a stricter selectivity in *Deinococcus radiodurans* also on both unmodified and 8-oxoG modified RNA.



Importantly, we found that all of the tested proteins have the ability to discriminate between 8-oxoG modified RNA and unmodified RNA- we are working on the mechanistic implications of these proteins in relationship to our regulatory RNAs. Since these are all newly characterized proteins, we expect that a manuscript will be written soon on their role in *D.radiodurans*. PNPase and DR_0335 showed the strongest binding ability to 8-oxoG modified RNA; this is interesting given that helicases have been strongly implicated in mechanisms of DNA repair surrounding radiation resistance.

Lastly, we have determined the phenotypic importance of PNP in the accumulation of 8-oxoG in *D. radiodurans*. As shown below, ELISA assays showed that 8-oxoG level was reduced in RNA

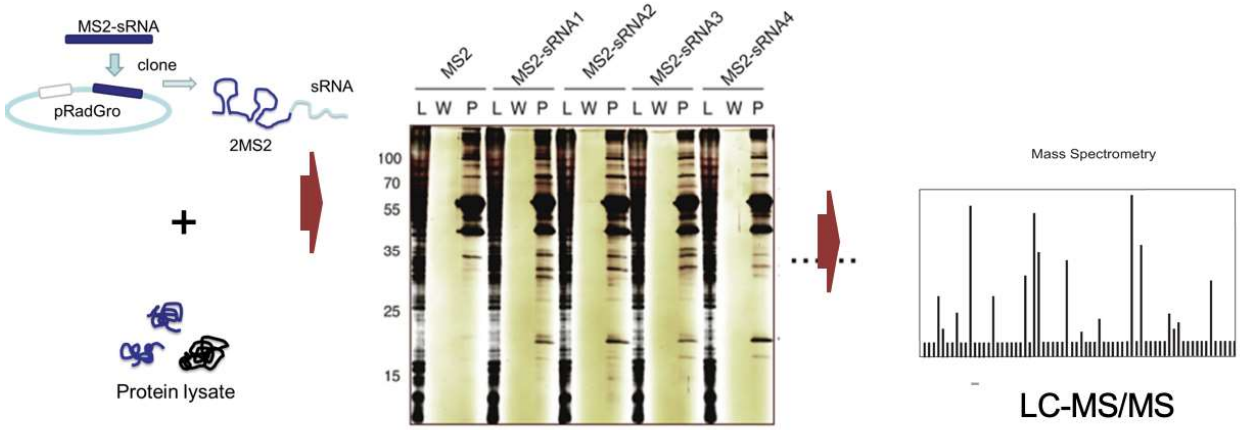
from PNPase knock-out strain compared to R1. For these experiments, total RNAs were extracted from R1 and pnp KO strains under 0-, 50- and 100-mM H2O2. RNA samples were nuclease and calf intestinal phosphatase. (we constructed successfully a PNP KP mutant and verified it) and determined 8OG levels using ELISA kit (Cayman Chemical). The ELISA was analyzed using a wavelength of 412nm in the BioTek Cytation3 plate reader. The final output values were divided by 0.38 to account for RNA cross-reactivity in the kit. The resulting 8OG concentrations, in pg/mL, were compared statistically using JMP 10 (SAS) with N=2 (representing the average value of each dilution, as technical replicates on
the ELISA).



This work is being prepared for publication.

9. Identification of a novel *D. radiodurans* protein (DR_2009) that bind regulatory sRNAs-identification of novel proteins that associate with regulatory small RNAs in *D. radiodurans.* To further understand proteins that contribute to the activity and function of the newly regulatory RNAs that we have uncovered and are currently characterizing in *D. radiodurans*, we conducted a pulldown assay to search for sRNA-binding proteins as this has not been done in *D. radiodurans* and therefore we ignore how regulatory RNAs that sense environmental stresses truly function in this organism.

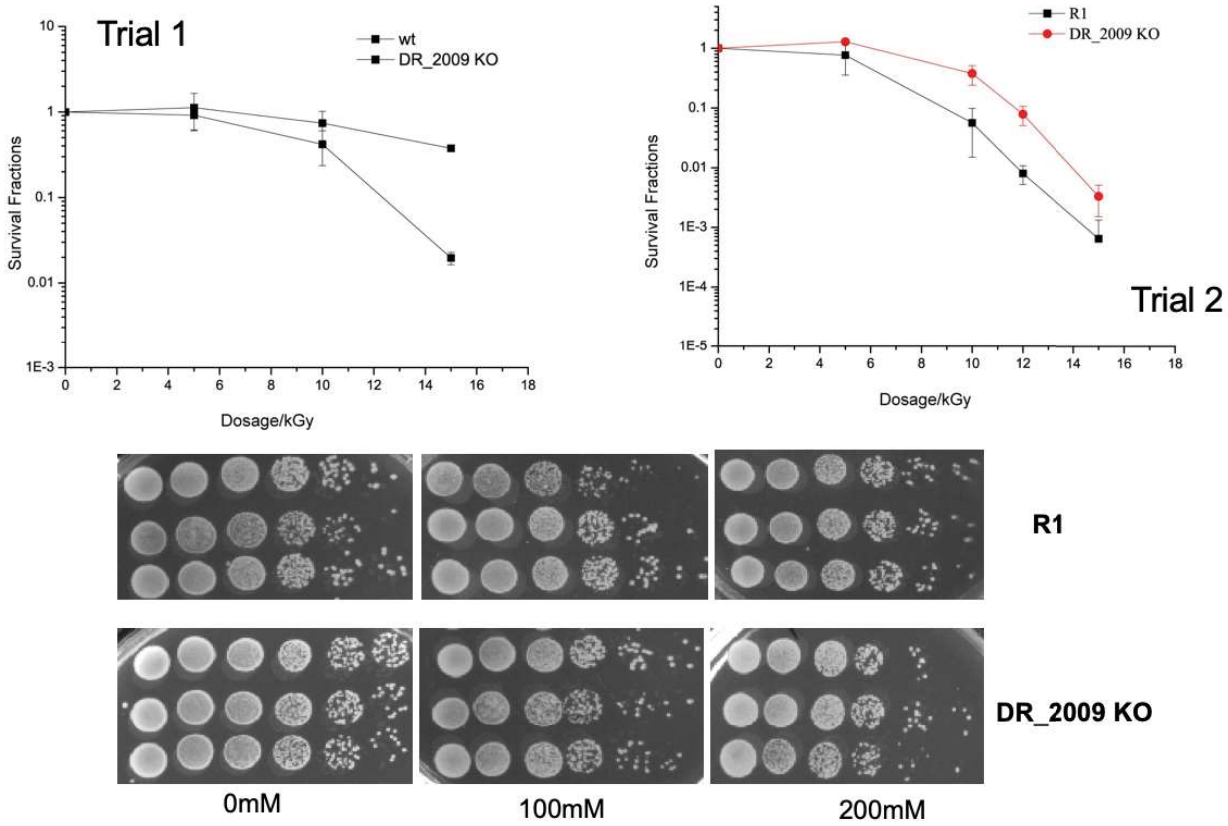
The assay is summarized below. Briefly, the sequences of MS2 protein binding sites (MS2BD) were added to the 5' end of the sRNA sequences and cloned into *D. radiodurans* with pRADgro plasmid. Mutants were cultured to exponential phase (OD_{600nm}= 1) and collected for total protein extraction. To find proteins associated with each sRNA in vivo, total lysates from *D.radiodurans* containing pMS2 and pMS2-sRNA were incubated with 2 µg of purified MS2-MBP protein for 1 hr at 4C. Proteins associated with each sRNA were eluted for LC-MS/MS analysis.



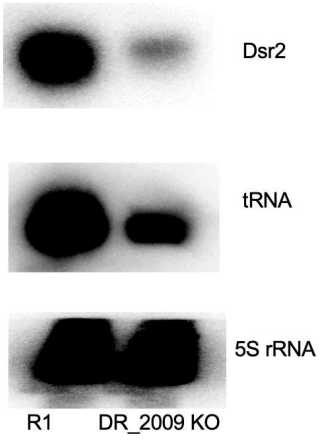
We conducted these pulldown assays with several regulatory RNAs (Dsr1, Dsr2, Dsr27, and Dsr30), to identify important proteins that contributed to a general protein binding mechanism. All the regulatory RNAs that we used for these assays were ones that we had demonstrated earlier to be relevant for responding to IR (that is, we have characterized that all these sRNAs naturally change expression levels under radiation). The results are on the table below: It is worth noting that DR_2009 is the only identified protein with RNA binding domain and it was enriched in samples from 3 of 4 the isolated RNAs:

Category	Name	Dsr1 Enrichment	Dsr2 Enrichment	Dsr27 Enrichment	Dsr30 Enrichment
Shared by 4 sRNAs	DR_0333	2.16	4.05	6.00	5.09
	DR_2287	2.15	2.37	3.77	3.30
Shared by 3 sRNAs	DR_1418	4.13	5.68	6.33	--
	DR_A0071	∞	∞	--	∞
	DR_2009	∞	∞	∞	--
Shared by 2 sRNAs	DR_2454	--	∞	--	∞
	DR_1455	--	2.57	--	5.55
	DR_2079	2.36	--	--	2.19
	DR_A0242	--	2.30	--	2.36

After focusing on the DR_2009 RNA binding protein and making a knock out strain of this protein, we have also realized that it affects the bacterial survival under acute radiation but not H2O2 (data shown below).



We have also uncovered that this protein affects levels of the regulatory RNAs in the cell (and even tRNAs). We have verified this with Dsr2 (one of the radiation sensory RNAs that we have uncovered and best characterize to date); we are currently testing the effect of this protein on the levels of other relevant regulatory RNAs. Data is shown below; 5s rRNA is a loading control that should not be affected.



TIMELY COMPLETION OF THE LAST TWO FINDINGS DESCRIBED INTO PUBILCATIONS WERE SIGNIFICATLY AFFECTED BY COVID-19 SHUTDOWN

IV. Dissemination of results

Publications (fully or partially supported by this award)**:**

1. J. K. Villa, Y. Su, L.M. Contreras and M. Hammond. "Synthetic Biology of Small RNAs and Riboswitches", *Book chapter*, in Papenfort, K, Storz, G (eds.), Regulating with RNA in Bacteria and Archaea, 6(3) (2018). ASM. Available from: doi.org/10.1128/microbiolspec.RWR-0007-2017.

2. A. Chen, L.M. Contreras, and Benjamin Keitz, "Imposed Environmental Stresses Facilitate Cell-free Nanoparticle Formation by Deinococcus radiodurans," *Applied and Environmental Microbiology*, 83 (18): e00798-17 (2017). Available from: doi:10.1016/10.1128/AEM.00798-17

3. J. Vazquez-Anderson, M. K. Mihailovic, K.C. Baldridge, K. Reyes, K. Haning, S. H. Cho, P. Amador, W. Powell, and L.M. Contreras, "Optimization of a novel biophysical model using large scale in vivo antisense hybridization data displays improved prediction capabilities of structurally accessible RNA regions," Nucleic Acids Research Journal, (2017). Available from: doi:10.1093/nar/gkx115.

4. J. K. Villa, Y. Su, L.M. Contreras and M. Hammond. "Synthetic Biology of Small RNAs and Riboswitches", Book chapter, in Papenfort, K, Storz, G (eds.), Regulating with RNA in Bacteria and Archaea, 6(3) (2018). ASM. Available from: doi.org/10.1128/microbiolspec.RWR-0007-2017.

5. M. K. Mihailovic, J. Vazquez-Anderson, Y. Li, P. Vimalathas, V. Fry, R.A. Lease, W. Powell, and L.M. Contreras. "High-throughput in vivo mapping of RNA accessible interfaces to identify functional sRNA binding sites." Nature Communications, 9:4084, 2018

6. A. Chen, M.W. Sherman, and **L.M. Contreras**. "Discovery and Characterization of Native *Deinococcus radiodurans* Promoters for Tunable Gene Expression". *Applied and Environmental Microbiology, AEM.01356-19 (2019).* Available from: doi.org/10.1128/aem.01356-19 **Paper featured in issue's cover.**

7. Han et al (under preparation) PNPase identification of oxidized RNAs under radiation stress in *D. radiodurans*

National Presentations, in addition to the yearly AFOSR technical meeting.

1.."Novel insights into understanding and designing regulatory RNAs," American Chemical Society (ACS) National Meeting, San Francisco, CA, April, 2017

2."Understanding and Engineering RNAs for Programmable Gene Control," University of Houston, Biology and Biochemistry Department, Houston, Tx, February 2016

3.“Assembling RNA modules to characterize responsive regulatory networks in vivo,” Gordon conference on RNA Nanotechnology, Ventura, CA, January 2017

4. ”High Throughput tools for understanding and rationally engineering regulatory sRNA,” Society of Biological Engineering (SBE) meeting, San Diego, CA, January 2016.

5. “Functional characterization and design of regulatory RNAs using novel high-throughput tools”-American Society of Biochemistry and Molecular Biology, San Diego, CA---April 22, 2018

6. “Discovery, characterization and targeting of regulatory RNAs that transduce extracellular stimuli into intracellular responses” University of Houston Medical School, Microbiology and Molecular Genetics Dept. –February 22, 2018

7.“Understanding and Engineering RNAs for programmable gene control” University of North Carolina Chapel Hill, Biology Dept. –January 16, 2018
.
8.“Better Living through microbes” Osher Lifelong Learning Institute, Austin, TX, November, 2017

9.“Novel insights into understanding and designing regulatory RNAs,” MIT, Boston, MA, November, 2017.

10. “Finding a needle in a haystack: parsing integrative systems data to define RNA-driven regulatory networks,” Manus Biosynthesis, Boston MA, November, 2017

11. “Rational sRNA design for strain engineering, Biochemical and Molecular Engineering , Engineering Conferences International (ECI), Newport Beach, CA, July, 2017

12. 2018 AIChE Annual Meeting, Pittsburgh, PA. "Utilizing native metabolic pathways in Deinococcus radiodurans for metallic nanoparticle biosynthesis" (oral)

13. 2018 Central US Synthetic Biology Workshop, Evanston, IL. "Engineering silver nanoparticle biosynthesis via sRNAs in Deinococcus radiodurans" (poster)

14.. “Understanding and Engineering RNAs for programmable gene control” Duncan And Suzanne Mellichamp Emerging Leader Lecture. University California Santa Barbara (UCSB) Santa Barbara, CA. Tuesday Nov. 17th, 2018,

15. “Tracking RNA modifications to understand early health effects of oxidative prone air” EpiBio Meeting. San Francisco, CA., October 5, 2018

16. “RNAs as sensors of our environment” invited speaker Deans Scholar Lunch, UT Austin” Sept. 28, 2018

17. “Novel insights into regulatory RNAs using novel high-throughput tools”, Case Western Reserve University (Chemistry Dept), Cleveland, OH, September 2018.

18. “Using synthetic biology to characterize and engineer RNAs”, Engineering Biology Research Consortium (EBRC), Fort Collins, CO, September 2018.

19. “Discovering, understanding and designing regulatory RNAs for biotechnology”, Biotechnology Symposium at Oregon State University, Corvallis, OR, June 2018.

20. 2019 EBRC Retreat, Boston MA. “Expanding synthetic biology tools through in vivo studies of regulatory RNAs in extremophiles” (oral)

21. 2018 Central US Synthetic Biology Workshop, Evanston, IL. “Discovery of Radiation-Responsive RNA Regulators in Deinococcus radiodurans" (poster)

22. Graduate and Industry Networking Event. February 3rd, 2019. Austin, TX. “Identification and Optimization of Promoter Sequences for Genetic Engineeringin the Extremophile, Deinococcus radiodurans.” (poster)

23. Novel insights into regulatory RNAs using novel high-throughput tools” University of Missouri”. University of Michigan (Chemical Engineering), April, 2019

24. “Novel insights into regulatory RNAs using novel high-throughput tools” University of Maryland Baltimore Country, UMBC, (Biochemistry Dept.), Baltimore, MD, March. 2019

25. “Novel High-throughput tools for in vivo functional characterization of regulatory RNAs” RNA-Protein interactions/Long/Noncoding RNAs Keystone Symposia Meeting, BC, Canada, February 2019

26. “Novel insights into regulatory RNAs using novel high-throughput tools” University of Missouri (Biochemistry Dept.), Columbia,MO February, 2019

27.Novel insights into regulatory RNAs using novel high-throughput tools” University of Maryland Baltimore Country, UMBC, (Biochemistry Dept.), Baltimore, MD, March. 2019

28. “Novel insights into regulatory RNAs using novel high-throughput tools,” University of Michigan (Chemical Engineering), April, 2019

29.“Biologically inspired sensors of nucleic acid assembly” Foundations of Nanoscience Meeting (“Chemical Tools for DNA Nanotechnology” Track), Snowbird, UT April, 2019

30. “Novel high-throughput tools for in vivo functional characterization of regulatory RNAs”. Riboclub Meting, Orford, Canada, September, 2019

31. “Understanding and engineering RNAs for programmable gene control,” Carnegie Mellon University, Chemical Engineering Seminar, October, 2019

32. “Using synthetic biology and machine learning tools to characterize and engineer RNAs,” University of Houston, Chemical Engineering Seminar, February, 2020
33. Understanding and engineering RNAs for programmable gene control,” Rice University, Biosciences Dept. Seminar, February, 2020

33.. “Tracking epitranscriptomics modifications to understand early health effects of oxidative-prone air”, Transportation, Air Quality, and Health Symposium meeting, Austin, Texas, February 19, 2019

34. GAIN 2020 | Identifying regulators of bacterial stress responses utilized in extreme environments | Presented by Jordan K. Villa | Feb 4th 2020 | Burnt Orange Award (1/4 given out of ~70 students)

35. GAIN 2020 | The effects of environmental stresses on the biosynthesis and properties of nanoparticles synthesized by *Deinococcus radiodurans* | Presented by Angela Chen | Feb 4th 2020 | Burnt Orange Award (1/4 given out of ~70 students)

***ALL OTHER INVITED TALKS WERE CANCELLED DUE TO COVID 19**

Collaborators in this work:

The following AFOSR collaborators made great contributions to our work:

(1) Warren Powell: We have published two papers and have one currently in review

(2) Howard Salis: We are have our first manuscript together from our work on modeling experimentally characterized post-transcriptional regulation under review

(3) Tom Lankin/Roland Saldahna- We have established a closer connection with Tom’s group and we have published one manuscript together. They have been instrumental in assisting us to get effective deletion protocols in our lab.

(4) Nancy Kelley-Loughnane- We continue to rely on this group for ideas exchange etc. given the common interest in regulatory/synthetic RNAs. They have recently contributed an article to the edition of Methods on RNA that Dr. Contreras has been leading

(5) Dr. Keith Keitz.